RESPONSE

To Mr. Akiteru Tamura, Examiner of the Patent Office

1. Indication of International Application

PCT/JP2004/004612

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5. Contents of response

(1) In the Written Opinion dated June 1, 2004 (mailing date), the Examiner cites the following documents 1 to 6 and holds that claims 1 to 16 lack an inventive step, claims 12, 13 and 17 lack novelty, claims 17 and 19 to 23 lack an inventive step and claims 24 to 27 lack an inventive step.

Document 1: Life Science Foundation (annual report), Heisei 14 edition (01 March, 2003) pp.17-19

Document 2: Cell.Mol.Life Sci., Vol.58, No.8, pp.1061-1066 (2001)

Document 3: Science, Vol.287, No.5457, pp.1489-1493 (2000)

Document 4: Gendai Kagaku Zokan 41, Saisei Igaku・Saisei Iryo (01 July, 2002) pp.24-28

Document 5: Proc.Natl.Acad.Sci.USA, Vol.98, No.23, pp.13090-

13095 (2001)

Document 6: FEBS Lett., Vol.475, No.1, pp.7-10 (2000)

In view of such view of the Examiner, the Applicant would respectfully present the opinion in the following. The Applicant believes that it is appreciated that the present invention has novelty and inventive step over the cited references (documents 1 to 6), from the statement described in detail below.

(2) The gist of the present invention

The present invention is as described in the claims.

That is, the invention of this application is characterized in that spermatogonial stem cells are grown by culturing the spermatogonial stem cells using a medium containing glial cell-derived neurotrophic factor (GDNF) or an equivalent thereto and leukemia inhibitory factor (LIF) in a method of growing spermatogonial stem cells. According to the method of the invention of this application, it is possible to grow spermatogonial stem cells *in vitro* for a long period, a task that has conventionally been impossible.

Furthermore, the invention of this application relates to spermatogonial stem cells grown using the above-described method, a therapeutic agent for infertility containing the spermatogonial stem cells, a method of producing sperm, embryos, non-human offspring and the like which comprises a step of growing spermatogonial stem cells by the above-described method, a method of producing sperm, a transgenic non-human animal and the like incorporating an exogenous gene by introducing the exogenous gene to spermatogonial stem cells grown using the above-described method, and the like.

(3) Comparison between the present invention and the cited references

(a) rejection of claims 1-16 for the lack of inventive step

Document 1 states that even when using the cell differentiation suppressive factor LIF in the cultivation and maintenance of ES cells, it is troublesome to maintain an undifferentiated state while retaining the capacity of multiple differentiation. However, this statement is concerned with the cultivation and maintenance of "ES cells", and there is no statement or suggestion concerning the use of LIF in the cultivation and maintenance of "spermatogonial stem cells" in document 1.

Additionally, document 1 states that an investigation using the seminiferous tubule transplantation method has been ongoing to determine whether spermatogonia maintained and proliferated with the addition of GDNF possess the function for stem cells. However, "spermatogonial stem cells" represent only a very small population among "spermatogonia" (usually accounting for about 1.25% of spermatogonia and for about 0.03% of germ cells); even if maintenance and proliferation of "spermatogonia" are observed as indexed by BrdU uptake rate (page 18, line 28) and the like, it is impossible to distinguish between "spermatogonial stem cells" and "spermatogonia other than spermatogonial stem cells" by the method, so that this does not mean maintenance and proliferation of "spermatogonial stem cells". Although the effect on the maintenance and proliferation of "spermatogonial stem cells" cannot be determined unless based on measurements of stem cell functions such as colonization by seminiferous tubule transplantation, document 1 only states that "an investigation has been conducted using the seminiferous tubule transplantation method", and does not show any result thereof.

On the other hand, the separately attached document Biology of Reproduction, Vol.68, p.2207-2214 (2003)

(hereinafter referred to as "reference A") discloses the results of an examination of the influences of various soluble growth factors on the maintenance of spermatogonial stem cells *in vitro*, based on the seminiferous tubule transplantation method. The same document states concerning LIF that "Transplantation results showed that none of these factors (including LIF) individually improved spermatogonial stem cell (SSC) maintenance in vitro, compared with the co-culture with STO feeders without any added factors (page 2210, right column, lines 37 - 40; see Fig. 3)."

Regarding GDNF, it is stated that "When donor cells were cultured with 100 ng/ml GDNF, the colony number was significantly greater than that for the control culture (- 1.6-fold increase, P=0.033; Fig. 3), although donor cell culture with 10 ng/ml GDNF did not result in a significant difference (- 1.2-fold increase, P=0.276; Fig. 3) (page 2211, left column, lines 25 - 30)" and that "...GDFN had a positive effect on in vitro maintenance of SSCs for 7 days (page 2211, left column, lines 32 - 34)". However, considering the fact that in a medium containing no additive factors, only 12% of SSCs remained in culture for 7 days (page 2209, left column, lines 25 - 26 etc.), 12%×1.6=only about 19.2% of spermatogonial stem cells were maintained even when using 100 ng/ml GDNF; it is evident that spermatogonial stem cells did not proliferate and rather decreased significantly.

Therefore, reference A teaches that spermatogonial stem cells cannot be grown even when using GDNF or LIF in the cultivation of spermatogonial stem cells *in vitro*, and in addition, there is no statement or suggestion concerning the use of GDNF or an equivalent thereto and LIF in combination in the cultivation of spermatogonial stem cells.

The Examiner has found that "in document 2, it is stated

that GDNF and LIF, a member of the IL-6 family, control the self-proliferation and differentiation of stem cells in spermatogenesis". However, no description is given that LIF controls the self-proliferation of stem cells in spermatogenesis. Additionally, although it is stated that "GDNF controls the self-proliferation and differentiation of stem cells in spermatogenesis", based on the results of an *in vivo* analysis in GDNF-gene-targeted mice and GDNF-overexpressing mice, there is no statement that GDNF stimulates the growth of spermatogonial stem cells *in vitro*. Furthermore, there is no statement or suggestion concerning the use of GDNF or an equivalent thereto and LIF in combination in the cultivation of spermatogonial stem cells.

Document 3 states that GDNF controls the self-proliferation and differentiation of undifferentiated spermatogonia, including spermatogonial stem cells. However, whether these "undifferentiated spermatogonia" are "spermatogonial stem cells" cannot be determined unless based on measurements of stem cell functions such as colonization by seminiferous tubule transplantation as described above; in document 3, stem cell functions were not measured. Additionally, it is stated that over all proliferation rate of spermatogonia was not enhanced (page 1491, left column, lines 32 - 33; Figs. 5A - C etc.) by GDNF overexpression, that a differentiation block rather than hyperproliferation probably causes the accumulation of spermatogonia (page 1491, left column, lines 34 - 36 etc.) by GDNF overexpression, and that Testicular tumors (consisting of undifferentiated cells) developed regularly in older GDNF-overexpressing mice (page 1491, right column, lines 7 - 8 etc.); these facts indicate that excess GDNF induces tumor due to inhibition of spermatogonia differentiation, rather than control of

spermatogonia growth. Thus, document 3 gives a rather negative suggestion about the effect of GDNF on spermatogonia growth, and includes a statement that hampers the idea of using GDNF for the purpose of growing spermatogonial stem cells *in vitro*.

Furthermore, in document 3, there is no statement or suggestion concerning the use of LIF in cultivation of spermatogonial stem cells or the use of GDNF or an equivalent thereto and LIF in combination.

Hence, none of documents 1 to 3 state that spermatogonial stem cells can be grown *in vitro* using GDNF or an equivalent thereto, or LIF; in view of reference A, we consider that it was thought impossible to grow spermatogonial stem cells *in vitro* even when using GDNF or an equivalent thereto, or LIF, with the state of the art of the time of filing of this application. Additionally, in these documents, there is no statement or suggestion concerning the use of GDNF or an equivalent thereto and LIF in combination in the cultivation of spermatogonial stem cells *in vitro*. Furthermore, the accomplishability of the remarkable effect of growth of spermatogonial stem cells over a long period of as long as 5 months by using GDNF or an equivalent thereto and LIF in combination cannot be easily arrived on the basis of documents 1 to 3 by those skilled in the art.

Therefore, we believe that the invention of claims 1 to 16 has an inventive step over documents 1 to 3.

(b) rejection of claims 12, 13 and 17 for the lack of novelty

The spermatogonial stem cells concerning claims 12, 13, and 17 are characterized in that they have been grown by culturing in a medium containing GDNF or an equivalent thereto and LIF.

On the other hand, document 4 states that the

spermatogenic capacity was acquired by transplanting spermatogonial stem cells to the testis, but the spermatogonial stem cells used therein do not have the above-described feature.

Therefore, we believe that the invention of claims 12, 13 and 17 has novelty over document 4.

(c) rejection of claims 17 and 19-23 for the lack of inventive step

The inventions of claims 17 and 19 to 23 include a step of growing spermatogonial stem cells by culturing the spermatogonial stem cells using a medium containing GDNF or an equivalent thereto and LIF, or spermatogonial stem cells grown by the step, as a feature that specifies the present invention.

As described in (a) above, the method of growing spermatogonial stem cells by culturing the spermatogonial stem cells using a medium containing GDNF or an equivalent thereto and LIF cannot be easily arrived on the basis of documents 1 to 3 by those skilled in the art. Also, as described in (b) above, in document 4, there is no statement or suggestion about spermatogonial stem cells grown by the method.

Therefore, we believe that the invention of claims 17 and 19 to 23 has an inventive step over documents 1 to 4.

(d) rejection of claims 24-27 for the lack of inventive step

The inventions of claims 24 to 27 include a step of growing spermatogonial stem cells by culturing the spermatogonial stem cells using a medium containing GDNF or an equivalent thereto and LIF, as a feature that specifies the present invention.

As described in (a) above, the method of growing spermatogonial stem cells by culturing the spermatogonial stem

cells using a medium containing GDNF or an equivalent thereto and LIF cannot be easily arrived on the basis of documents 1 to 3 by those skilled in the art. Also, as described in (b) above, in document 4, there is no statement or suggestion about spermatogonial stem cells grown by the method.

Documents 5 and 6 describe a method of producing a transgenic mouse by introducing an exogenous gene into spermatogonial stem cells using a retrovirus. However, in these documents, GDNF or an equivalent thereto, or LIF was not used in the cultivation of spermatogonial stem cells, nor is given any suggestion thereof.

Therefore, we believe that the invention of claims 24 to 27 has an inventive step over documents 1 to 6.

(4) Conclusion

As described in detail in the above, we believe it is appreciated that the present invention has novelty and inventive step over the cited references.

6. List of annexed document

(1) Reference A: Biology of Reproduction, Vol.68, p.2207-2214 (2003)